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Influenza A virus in natural and artificial environments

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Science is facts; just as houses are made of stone, so is science made of facts; but a pile of stones is not a house, and a collection of facts is not necessarily science.

- *Jules Henri Poincaré*

To my near and dear

Abstract

Influenza is caused by influenza A virus, a single stranded RNA virus of the orthomyxoviridae family. In humans, it causes yearly outbreaks with high morbidity and excess fatality rates as a direct effect. Placed in its ecological niche however, in dabbling ducks, avian influenza virus (AIV) induce quite mild disease. It is when the virus crosses the species barrier that pathogenic traits are attributed to infection. Also infection of close relatives to dabbling ducks, the domestic chicken, cause morbidity and may in some cases change the virus into a highly pathogenic variant with nearly 100% fatality rate. Being a very adaptable virus, these spill-over events are frequent, and numerous species are susceptible to influenza virus. When a subtype of influenza which has not previously infected humans crosses the species barrier, adapts to humans and spread easily, a pandemic event is imminent. There is no cure for influenza infection, and vaccination is a cumbersome endeavor, so currently the strategy when a pandemic strikes is damage control.
In this thesis, I have been involved in a surveillance project, to increase our knowledge of how influenza travels across the globe with its natural host. We have also used animal models to investigate the pathological effects in mallard ducks and their susceptibility to re-infection. Furthermore, we have evaluated the effect and the potential risk of frivolous use of the anti-viral agent oseltamivir, and also investigated a novel approach to the classic virus isolation method of growing virus in embryonated chicken eggs (ECE's).

Indication was found in northern Alaska that prevalence of influenza is probably not lower here than in other breeding areas for dabbling ducks, as has been previously suggested. As these birds travel over the Bering Strait, the reason for the genetic isolation of Eurasian and North American influenza A strains remains unclear.
Inoculation of mallards equipped with subcutaneous data transmitters indicated very little effect on the host and no stress above background level, and all birds gained weight throughout the trial. Only in four of six birds (65%) could a small temperature increase related to infection be recorded. However, more studies in a natural environment need to be conducted, to discern whether this variable is associated with an ecological cost as the captive trial ducks had access to food *ad libitum*.
The most commonly used anti-viral drug, oseltamivir, is poorly degraded in sewage plants and surface water, where dabbling ducks forage. Extensive use of the drug thus increases environmental levels of the active metabolite, oseltamivir carboxylate (OC). We show that mallards inoculated with A/H1N1 in an OC enriched environment generates resistant virus sporadically at OC level found today. Higher level of OC caused the resistant subspecies to dominate the virus population, which is not desirable in the influenza reservoir. An introduction of a OC-resistant pandemic virus to the human population would render the only antiviral defense toothless.
Isolation of influenza virus is traditionally performed by inoculation of infectious material into embryonated chicken eggs. As the chicken host is known to induce changes in AIV, we compared isolating and passaging two viruses both in ECE's and embryonated mallard eggs. Both egg species induced mutations in the primary passage, with no furthers changes in subsequent passages. Only in ECE's did one virus maintain wild-type configuration before one mutation was observed. Mallard eggs can based on these results not be advocated as preferable to ECE's when isolating and passaging AIV.

Original papers in this thesis:

This thesis is based on the following original publications, which will be referred to in the text by their roman numerals:

I. **Gene segment reassortment between American and Asian lineages of avian influenza virus from waterfowl in the Beringia area.**

Wahlgren J, Waldenström J, Sahlin S, Haemig PD, Fouchier RA, Osterhaus AD, Pinhassi J, Bonnedahl J, Pisareva M, Grudinin M, Kiselev O, Hernandez J, Falk KI, Lundkvist A, Olsen B.
Vector borne and zoonotic diseases 2008, 8(6) 783-790

II. **Mallard or chicken? A comparative study of influenza isolation on embryonated eggs from different species.**

Wahlgren J, Latorre-Margalef N, Sahlin S, Waldenström J, Falk K, Olsson G, Olsen B, Lundkvist Å.
Submitted

III. **Influenza virus in a natural host, the mallard: experimental infection data.**
Jourdain E, Gunnarsson G, Wahlgren J, Latorre-Margalef N, Bröjer C, Sahlin S, Svensson L, Waldenström J, Lundkvist A, Olsen B.
PLoS One 2010 5(1): e8935

IV. **Environmental levels of the antiviral drug oseltamivir induce development of resistance mutation H274Y in influenza A(H1N1) virus.**
Järhult J.D, Muradrasoli S*, Wahlgren J*, Söderström H, Orozovic G, Gunnarsson G, Bröjer C, Latorre-Margalef N, Fick J, Grabic R, Lennerstrand J, Lundkvist Å, Waldenström J, Olsen B.
Submitted

Abbreviations

AA	Amino acid
AIV	Avian influenza virus
ECE	Embryonating chicken egg
GAL	Galactose
HA	Hemagglutinin
HPAI	Highly pathogenic avian influenza
LPAI	Low pathogenic avian influenza
NA	Neuraminidase
NS1	Non-structural protein 1
NS2/NEP	Nuclear export protein
OC	Oseltamivir carboxylate
PCR	Polymerase chain reaction
PKR	Protein kinase R
RT	Reverse transcription
SA	Sialic acid

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1 Introduction

Novel introductions of influenza viruses into the human population from the animal kingdom continue to be a major health problem worldwide. Historically, we have been ill-prepared for pandemics that strike fast and with no warning, and the notion of influenza as a disease that comes from animals is fairly new. During the last decade, influenza research has intensified to previously unseen magnitudes, and together with this, also the interest in the inclusive virology research that focuses on influenza not only as a human pathogen but as an entity that is part of an ecosystem. With an increased knowledge of the dynamics of the ecosystem, and the interplay of the parts therein, the goal is for human science to be able to more accurately predict new pandemics and take appropriate preventive countermeasures.

1.1 Background

Placed in its ecological niche, commonly in dabbling ducks, influenza A virus is a benign disease (Jourdain, Gunnarsson et al. 2010). However, it is a very adaptable virus and it has been able to infect, and adapt to, a wide range of hosts (Webster, Bean et al. 1992). The disease associated with infection shows a broad range of symptoms, depending in part on the genetic properties of the virus, but also on which species of host is infected (Horimoto and Kawaoka 2005; Kishida, Sakoda et al. 2005; Isoda, Sakoda et al. 2006). In the natural host, no signs of infection can be identified by ocular inspection, while other bird species and mammals are more severely affected with symptoms ranging from very mild to very severe and ultimately death. It was first identified as an animal disease in 1878, when Eduardo Perroncito described a disease affecting poultry in northern Italy. Observations from this study describe an easily transmitted, initially mild disease which increased in pathogenicity over time and in the end killed virtually all domestic fowl in the area. “Fowl plague”, as the disease would be called, was proved to be a viral disease in 1901 but not identified as influenza virus until 1955 (Capua and Mutinelli 2001). Since it was first described in 1878, highly pathogenic avian influenza (HPAI) virus has caused 21 documented outbreaks of fowl plague between 1959 and 2003 (WHO 2004).

As a human disease, it is hard to know when it was first introduced to the human population, or when it became truly endemic. Epidemics that may well have been influenza have been described, more or less accurately, by physicians for over 2000 years. The first verifiable influenza pandemic however, is the Russian flu of 1889-1892 (Nicholson 1998). Since,

several pandemics of varying severity have struck the world, each deriving from an introduction of a novel virus, or parts thereof, from the animal kingdom (Kawaoka, Krauss et al. 1989). The most sinister example of how horrific an introduction of an easily transmitted virus may be to a naïve human population is the 1918 pandemic, commonly known as the "Spanish flu". It swept across the world in three waves, increasing in virulence each year, and leaving over 20 million people dead in its tracks (Erkoreka 2010). As the world watched, appalled by the effects of the disease, intense research was initiated to understand the causative agent. It would not be until 1933, however, that a filterable substance was isolated which induced influenza-like symptoms in humans and was easily transmitted between ferrets (Smith 1933). Kochs postulate was later fulfilled after the influenza virus could be isolated from the throat of one of the team members after having been sneezed upon by one of the ferrets and subsequently developed influenza symptoms (Nicholson 1998). Despite thorough research where the molecular functions of the virus have been investigated in detail, influenza A virus continues to be a common human pathogen and each year the endemic, seasonal flu, results in mortality peaks and wide-spread morbidity with vast economic consequences (Franco-Paredes, Hernandez-Ramos et al. 2009). Rather than having been able to control this infection, we have adapted ourselves and our behaviour to minimize its damage when the flu season strikes (Robinson 1990; Webster 2002). Random introductions of novel viruses from the animal kingdom also continue to be a major health- and economical problem for the human population, and several pandemic events have occurred during the 19th and 20th centuries, including the notorious "Spanish Flu" (Hope-Simpson and Golubev 1987; Del Rio and Hernandez-Avila 2009; Morens, Taubenberger et al. 2010). It is believed that this first recorded pandemic was the result of a direct transmission of a highly pathogenic avian virus to humans, without intermediate hosts, although this belief has recently been called to question (Reid, Fanning et al. 2004; Antonovics, Hood et al. 2006). Later pandemics did not occur in the same direct fashion but used pigs, which are permissive to both avian and human adapted viruses, as mixing vessels (Ito, Couceiro et al. 1998). Until 1977 and the "Russian flu" pandemic, each time a new subtype emerged and spread globally it replaced the previously circulating strain of influenza virus (Bean, Cox et al. 1980).

1.1.1 Influenza A virology

Influenza A virus belongs to the *Orthomyxoviridae* family together with influenza B, influenza C, isavirus and thogotovirus (Murray 2009). It is a pleomorphic virus containing eight gene segments, and the virion is made up by the interior matrix (M1) protein and the

nucleocapsid, consisting of viral RNA, nucleoprotein (NP) and the three polymerase proteins making up the transcriptase (Murray 2009). The nucleocapsid is enveloped by a host-derived membrane containing three viral proteins; hemagglutinin (HA), neuraminidase (NA) and matrix 2 (M2). Classification and the nomenclature of influenza A viruses is based on what type of HA and NA is present in the membrane (WHO 1980). There are to date 16 serologically distinct HA types, and nine different NA types described (Fouchier, Munster et al. 2005).

1.1.2 Nomenclature

Each virus is named after its serological phenotype, starting with determining if it belongs to influenza A, B or C, after which place of sampling/isolation is stated, then serial number of the sampling protocol and year (WHO 1980). Last the subtype is stated in parenthesis. Example: A/Sweden/937/09 (H5N2). When a virus is isolated from a species other than human, it is specified between serological type and geographic location of the sampling. Example: A/Mallard/Sweden/937/09 (H5N2).

1.1.3 Replication

The wide range of hosts to influenza A virus and its ability to adapt to new species, may in part be due to the variability of its genome. The genome consists of eight single-stranded, negative sense RNA segments (Lamb and Choppin 1983). Single stranded genomes allow for high mutation rates, as there is no second strand that can otherwise be used for proof-reading (Webster, Shortridge et al. 1997). Errors typically occur during transcription at a rate of $1/10^3$ to $1/10^4$ nucleotides for single stranded genome viruses versus $1/10^8$ nucleotides in double stranded DNA viruses (Holland, Spindler et al. 1982; Duffy, Shackelton et al. 2008). Unlike for complex, large genome species, this is a beneficial trait as it can help the virus to adapt quickly, should a new environment present a different selection pressure. Having a segmented genome of influenza A also allows for another way to change its composition, i.e antigenic shift. This may occur if one cell becomes simultaneously infected by two different influenza A viruses (Hinshaw, Bean et al. 1980). As many as 256 different variants may then be formed through reassortment of the different segments.

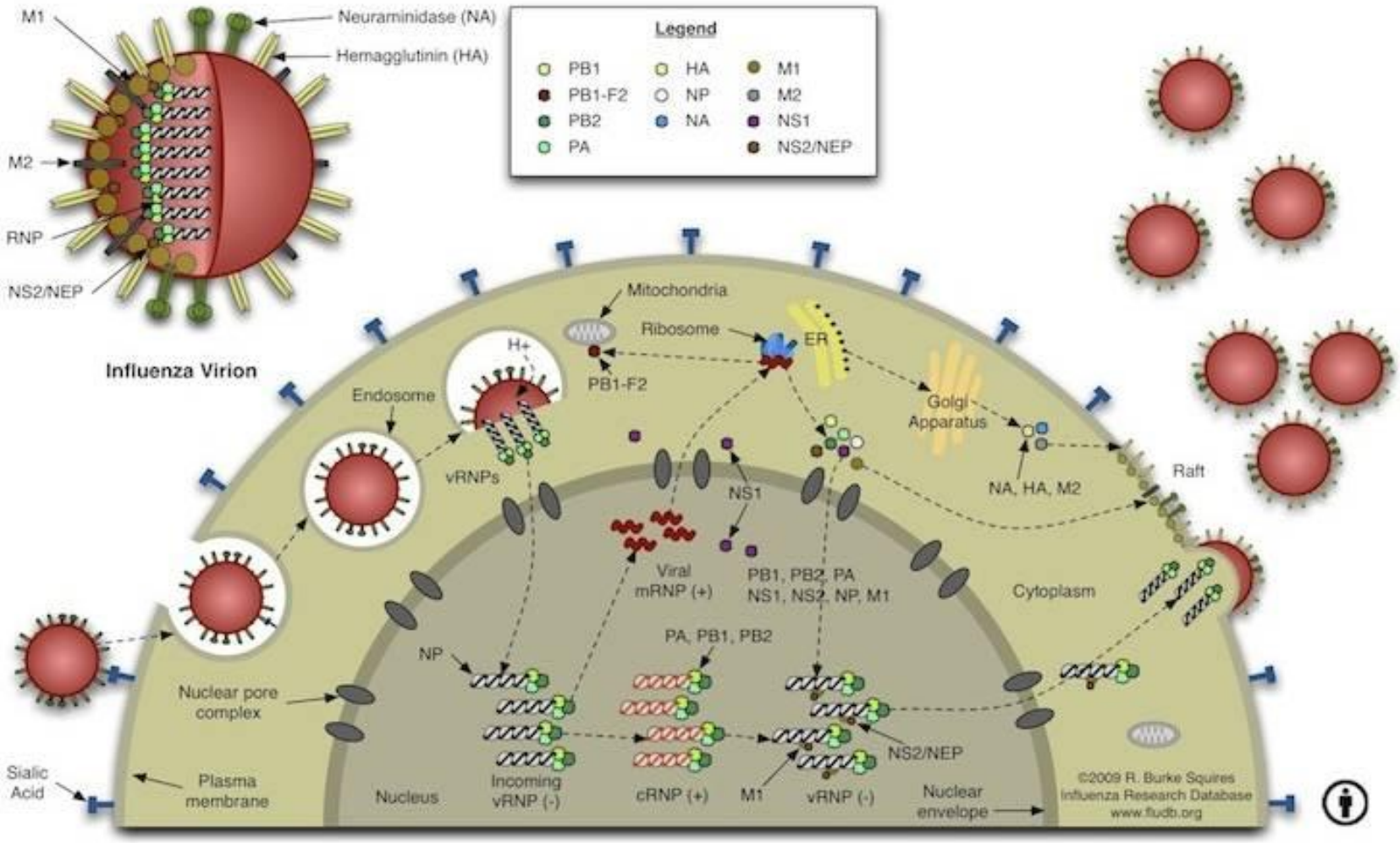

Fig. 1
Schematic image of the influenza A replication cycle in the cell. Courtesy to Cold Spring Harbor Laboratory (CSHL), Ontario Institute for Cancer Research (OICR) and the European Bioinformatics Institute (EBI)

1.2 Virus structure and function

The eight gene segments code for ten or eleven proteins, depending on the presence of an alternative reading frame in one of the polymerase genes. This alternative reading frame gives rise to the protein now known as PB1-F2, able to induce apoptosis in cells by aiding in the release of cytochrome C from the mitochondrial membrane (Chen, Calvo et al. 2001). However, this extra reading frame is not present in all influenza A viruses, and the essential genes for virus infectivity and production are ten: HA, NA, matrix 1 (M1), M2, nucleoprotein (NP), non-structural protein 1 (NS1), nuclear export protein (NS2/NEP) and three polymerase subunits PA, PB1 and PB2 (Knipe 2007).

HA and NA mediates virus entry and release respectively, and M2 is a pH dependant ion channel (Sugrue and Hay 1991). The M1 protein is located under the viral envelope and interacts with the ribonucleoprotein (RNP) complex (Murti, Brown et al. 1992). It is widely accepted that the function of the protein is to provide protection to the RNPs and to give the virus rigidity and structure. Evidence for this plausible function, however, has proven difficult to obtain (Knipe 2007). The non-structural proteins NS1 and NS2/NEP have dynamic functions for virus replication and survival post infection. NS1 is expressed abundantly in infected cells, and has multiple functions. It binds to double-stranded RNA to prevent association with cellular protein kinase R (PKR) that would otherwise recognize it as foreign and trigger the innate immune response, which is further discussed under 1.3.1 (Lu, Wambach et al. 1995; Hatada, Saito et al. 1999; Williams 1999; Kuiken, Holmes et al. 2006). It is also responsible for diverting cell translation and the suppression of the host cell's post-transcriptional processing of mRNA (Geiss, Salvatore et al. 2002; Hilleman 2002; Baigent and McCauley 2003). NS2/NEP plays a vital role in virus replication, regulating the export of RNP complexes from the nucleus and the relative transcription of the influenza gene segments (Robb, Smith et al. 2009).

Genome segment	Proteins coded	Main function
1	PB2	Sub-unit of RNA-polymerase
2	PB1	Sub-unit of RNA-polymerase
	PB1-F2	Function unknown, pro-apoptotic
3	PA	Sub-unit of RNA-polymerase
4	HA	Viral attachment and membrane fusion
5	NP	Major structural component
6	NA	Release of new virions by preventing aggregation
7	M1	Facilitating migration of viral RNP in cell
	M2	Ion channel involved in uncoating of virus
8	NS1	Post-transcription modulation, interferon antagonist
	NS2/NEP	Mediates nuclear export of vRNAs

Table 1. Influenza A virus components and their main function

1.2.1 Entry

The surface protein HA allows virus to attach to sialic acid (SA) receptors on the host cell surface (Knipe 2007). This event triggers a cell-mediated endocytosis, and the virus find itself enclosed in an endosome (Dales and Choppin 1962; Patterson, Oxford et al. 1979). Endocytosis is an intrinsic mechanism of eukaryotic cells used to ingest and digest material from its surrounding. After endocytosis, the endosome undergoes a functional transition to become a lysosome, which degrades and digests the material within (Alberts B 2002). Among other changes in this transition such as the import of digestive proteases, the interior pH is lowered, which is of importance for influenza virus replication. As pH decreases in this environment, the HA surface protein undergoes a structural change that enables interaction with the endosome membrane, to fuse it with the virus membrane and release the RNA-RNP constructs into the cytoplasm (Matlin, Reggio et al. 1981; Stegmann, Morselt et al. 1987; Skehel and Wiley 2000). For this critical fusion step to take place, however, the native structure of HA must be changed. When HA is expressed by a virus-producing cell, it is in its native, non-infective form, HA_0. Post-translational cleavage of the protein into HA_1 and HA_2 is necessary for the endocytosed virion to fuse its membrane with that of the endosome (Scholtissek 1986). The cleavage of HA is a controlled mechanism which can only be performed by a specific enzyme that is not expressed in all cell types, which limits infection to certain tissues, though exactly what tissue is infected varies between species (Nagai 1993; Nagai 1995; Kido, Murakami et al. 1999).

1.2.2 Translation

Upon release of RNP complexes into the cytoplasm, the NP protein interacts with the cellular transport protein importin α, with the result of RNP complexes being imported into the nucleus (Martin and Helenius 1991; O'Neill, Jaskunas et al. 1995). Viral RNA is then transcribed into positive sense RNA by the virus' own RNA-dependant polymerase. This positive sense RNA can then be used for transcribing new negative sense vRNA, or be exported as mRNA to the cytosol where it is translated into viral proteins. Viral surface proteins will be translated through the golgi apparatus and transported to the cell surface and ultimately form dense clusters in the lipid rafts of the cell membrane (Scheiffele, Roth et al. 1997; Barman and Nayak 2000). The other proteins will be transported into the nucleus and form new nucleocapsids together with the new vRNA.

1.2.3 Assembly and budding

Assembled nucleocapsids containing negative sense vRNA and the core proteins are exported from the nucleus in a regulated manner, and transported to the membrane under the lipid rafts which contain clusters of viral surface proteins. Cellular exocytosis of the nucleocapsids will then form complete progeny virus with an envelope derived from the cellular membrane (Suomalainen 2002). However, as HA can bind SA receptors immediately, progeny virus will stick like glue to the host cell, and only when NA cleaves the SA is the virion released from the cell surface (Gottschalk 1957; Mitnaul, Castrucci et al. 1996).

1.3 Transmission

The influenza A virus, as other viruses, cannot replicate outside a host cell. In order to infect new individuals it needs to persist for some time outside a host organism. It seems that the influenza A virus is well adapted to persist in water. Under experimental conditions, avian influenza A virus strains stored in distilled water at +28 °C could remain infective for 100 days, at 17 °C for 200 days and possibly for as long as 1000 days at +4 °C (Stallknecht, Shane et al. 1990). However, under natural conditions, persistence of active virus is limited by the effects of pH, salinity, UV-radiation and presence of biologically active material such as degrading enzymes, bacteria and other microorganisms. Human influenza A virus strains are stable at a pH from neutral to 8.5, and infectivity decreases rapidly below pH 6.0. Avian influenza A virus strains exhibit more stability than human influenza A virus strains and can persist and remain active at pH 4.0 whereas human isolates do not persist pH below 5.0 (Webster, Yakhno et al. 1978). Infectivity is inversely related to salt content of water for

avian influenza A virus (Stallknecht, Kearney et al. 1990). In open air, human strains of influenza A virus can spread as an aerosol. The persistence and infectivity of these strains in open air is promoted by low humidity. Aerosols containing influenza A virus may remain infective for up to 24 hours or more at low humidity but only for an hour at high humidity (Hemmes, Winkler et al. 1960). Other limiting factors in open air are UV-radiation and wind. Some strains of influenza A virus can also be spread via fomites on hard surfaces such as stainless steel, where it can survive for up to two days (Bean, Moore et al. 1982). Thus many factors determine the suitability of different environments for persistence, infectivity and transmission of influenza A virus.

1.4 Clinical picture of human infection

Influenza A virus infection is most often a self-limiting disease with abrupt onset of high fever, malaise, cough and head and muscle ache. It causes symptoms for up to 2 weeks and requires on average 3-4 days of bed rest. The disease can be severe and sometimes lethal in young children, the elderly, those who are under immune suppression and people with underlying illnesses such as cardiac disease or asthma (Mandell, Douglas et al. 2005). A central question is how an infection essentially localized to the respiratory tract can produce such severe constitutional symptoms. As in many other infectious diseases, it is the immune response that contributes substantially to the clinical signs and symptoms in influenza and finally to the control of infection. These immune mechanisms can lead to both localized as well as systemic effects, i.e the local inflammation of the upper respiratory tract and the systemic muscle ache. However, in the case of highly pathogenic influenza viruses, the specific tropism for the upper respiratory tract is lost. The full mechanism for this characteristic remains unclear, but can in part be explained as the cleavage site of the HA protein is extended by several basic amino acids (Kuiken, Holmes et al. 2006). This allows for ubiquitous cleavage and this activation of the virus, and an unrestricted infectivity of to all cells to which the virus can bind. Other factor that enables the virus to enter the blood stream, survive, and exit at novel sites are less well known (Smith and Sweet 1988). Another factor is a deficiency in NS1, resulting in hyper inflammation, which is further discussed under subheading 1.5.3. Systemic infection and a hyper induced immune system combined can cause shock and multi organ failure, which defines the highly pathogenic variant of influenza. To date, only subtypes H5 and H7 have been able to become highly pathogenic, and trials have shown that only the introduction of a polybasic cleavage site may not be enough for other subtypes to become highly pathogenic (Steinhauer 1999; Stech, Veits et al. 2009). The

disease in the confirmed cases has been severe, often fatal. The most common symptoms have been high fever, lower respiratory tract symptoms of pneumonia progressing to acute respiratory distress, and leucopenia has been a common laboratory finding. Some cases have presented atypical symptoms such as diarrhea, vomiting, bleeding from the nose and gums and encephalopathologic signs.

1.5 Immunity and defense

To defend ourselves against the multifaceted fauna of microbes we are frequently assaulted by, we have quite impressive defense mechanism: the immune system. Parasites, bacteria and viruses have very different biological properties, and different strategies and targets inside the host organism. It is thus imperative for the immune system to be able to recognize a wide array of potential threats, and to adapt to the micro-fauna of our immediate surroundings. In general terms, the immune system can be divided into two main categories with regard to memory function. 1: The innate response, which is rather unspecific, but with an immediate reaction to the infection. 2: The adaptive immune response, which takes longer to react, but has the ability to remember chemical features of a pathogen and to respond more quickly and strongly the second time the same pathogen is encountered. These features are common to all vertebrates, and though there are minor differences in cell appearance and peptide sequences, discussions on mechanisms driving immunity can be extrapolated from human biology to any vertebrate (Pastoret 1998; Erf 2004).

1.5.1 Innate immune responses

As the name implies, the innate immune system are immediately ready to react to and combat pathogens without prior exposure. The mechanisms in the innate immune response are many and complex, but they all share the direct action and lack of memory. Most prominent in the non specific recognition of pathogens is the innate immunity's ability to discriminate "self from non-self", but apart from this, the innate immune response can react to general markers of many pathogens, like the cell surface lipopolysaccarides and flagellin of pathogenic bacteria or double stranded RNA of viruses and unmethylated bacterial and viral DNA. Cells of the innate immune system can quickly release pro-inflammatory cytokines like interferon and TNF-α to promote leukocytes to migrate to the area and stimulate pathogen clearance. Leukocytes are specialized in the ingestion and digestion of foreign particles, and they also use the degraded parts of whatever they just destroyed to stimulate the adaptive immune response through antigen presentation. The adaptive response is thus dependant on the innate

response, but not vice versa. When cells have been activated by interferon, they remain in that state for a period of time during which their resistance to new infections is markedly increased. In the case of influenza infection, it has been shown that an activated innate immune response can be protective for re-infection, both of homo- and heterotypic strains of influenza A virus (van der Goot, de Jong et al. 2003; Furuya, Chan et al. 2010).
As a last resort, every cell in the body has the ability to kill itself in a controlled manner when its mechanisms become hijacked, via apoptosis. Apoptosis can be induced by extracellular factors, or by innate systems in the cell itself (Takizawa, Matsukawa et al. 1993; Hinshaw, Olsen et al. 1994).

1.5.2 Adaptive immunity

When the adaptive immune response is activated, antibodies are produced with high specificity and affinity to the pathogen. Often there are, during a primary infection, only a small number of antibody producing cells that are able to be activated to a pathogen, and this response is amplified by cell division. Memory is the prominent feature of adaptive immunity, and once sensitized to a specific peptide, or part of a protein deriving from an infectious agent, memory cells retain their ability to produce highly specific antibodies to the pathogen, but stay in a dormant state. Upon a secondary infection, the response from memory cells is faster and much stronger than the response from naïve cells. Thus the immune system is able to neutralize the assault faster and more efficiently after the first time the pathogen is encountered. In fact, it is so efficient that the host often doesn't even get sick before the infection is cleared. The function of antibodies can vary, from directly assaulting a pathogen with a complement system that forms pores in the pathogen, causing water influx and lysis, to function as a flag for phagocytic cells or the direct neutralization of viruses by covering them and so physically blocking them from interacting with their potential targets (Pastoret 1998).

1.5.3 Viral countermeasures

For a virus to survive and propagate over time, it needs to overcome the immune system's mechanisms of viral control and clearance. It can achieve this actively by interacting directly with components of the immune system and manipulate its action, or passively by preventing its recognition in the first place (Wang, Li et al. 2000).

Innate immune manipulation

Being a negative-stranded RNA virus, influenza A is a potent inducer of the innate immune response, and the symptoms from inflammation could be worse still. However, the virus has specific mechanisms to prevent this. One of the NS1 protein's main features is that it inhibits the cell's ability to produce interferon, a potent pro-inflammatory cytokine and one of the first to be released by the innate immune response upon infection (Pauli, Schmolke et al. 2008). Newly synthesized viral RNA is complementary to the template and forms double-stranded RNA, which is not a normal feature in the cell. PKR is a cellular protein able to identify and respond to double-stranded RNA, and trigger a cascade of anti-viral protein expression. The NS1 protein is specialized in sequestering the double stranded RNA, preventing recognition and thus limit or inhibit the response. In the case of highly pathogenic influenza viruses, a mutation in the NS gene may be partly responsible for the increased pathogenic properties (Cheung, Poon et al. 2002). By failing to repress the early cytokine expression, the virus triggers a strong cytokine response, and combined with a systemic infection the inflammation becomes much more severe than during a local infection of a human adapted influenza virus (de Jong, Simmons et al. 2006; Kash, Tumpey et al. 2006). NS1 also prolongs virus production by inhibiting apoptosis in infected cells (Zhirnov, Konakova et al. 2002).

Escape from adaptive immunity

Where escape from innate immune responses involves association of NS1 newly produced double-stranded RNA, escape from the adaptive immune response is less direct. There are two ways for influenza A viruses to escape adaptive immunity, both of which ultimately changes the coating of the virion to render it unrecognizable by neutralizing antibodies, but through different mechanism. Primarily, and constantly ongoing, is the *antigenic drift*. As the single stranded RNA genome allows for a high mutation rate and rapid evolution, the virus will automatically be less recognizable by antibodies as the structure of the surface proteins change over time. Secondly, with instant major antigenic change is the *antigenic shift*. Antigenic shift occurs when one cell becomes infected by two different subtypes of influenza. The segmented genome allows for random combinations of the genetic setup as progeny virus is assembled, with as many as 255 new combinations as the result (Bouvier and Palese 2008).

1.6 Treatment strategies

To counteract the virus' ability to escape and repress the immune system there are two approaches to lessen the burden of influenza infection in humans. Primarily there is the preventive approach, using vaccines to prepare the body for infection and priming the adaptive immune system as though it had been previously infected. This is naturally the method of choice as it prevents an infection from taking hold. However, once infected, receiving vaccine does not affect the outcome of the disease and does not alleviate the symptoms. Should this happen, there are antiviral substances that directly hinder the viral replication, shorten period of morbidity and decrease mortality.

1.6.1 Vaccines

Vaccination against a disease utilizes the adaptive immune system's ability to remember a pathogen. By exposing the immune system to only a part of a pathogen, along with an inflammatory agent called an adjuvant, the immune system is stimulated to associate the foreign agent to a physical assault. The body then responds as it would to a natural infection, producing antibodies, memory cells and thus immunity to an assault by a pathogen with the same recognition sites that were used in the vaccine (Goldsby 2003). In the case of influenza A virus, the surface protein HA is used in the vaccine, causing the body to form neutralizing antibodies to the protein that otherwise would adhere to the target cell surface (Ruben 1990).

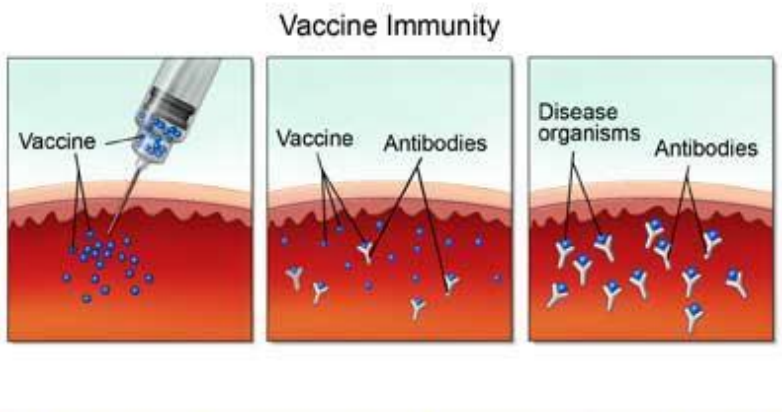

Fig 2. Illustration of vaccination and immunity. Injection of an antigen enables the immune system to produce antibodies able to recognize and neutralize a pathogen carrying the same antigen used in the vaccine.

1.6.2 Antiviral drugs

Antiviral substances directly interact and inhibit specific steps in the virus' life cycle and slow or completely arrest its propagation (Murray 2009). To date there are four antiviral drugs for influenza virus, two of which (oseltamivir and zanamivir) target the NA protein, and the other two (amantadine and rimantadine) the M2 protein. Amantadine and rimantadine interacts at an early stage of the replication by blocking the M2 ion channel, which prevents the ion influx and the disassembly of the nucleocapsid (Pinto and Lamb 2007). When M2 is blocked, the M1 protein fails to disassociate from the RNPs prior to the membrane fusion event, and M1-associated RNPs are released into the cytosol, and transport of RNPs into the nucleus is blocked (Martin and Helenius 1991). In the case of NA, oseltamivir and zanamivir inhibit the neuraminidase enzymatic activity. It does not prevent the virus from infecting cells or its replication, but inhibits the release of progeny virus (He, Massarella et al. 1999). These drugs are more benign to the patient, and are not associated with side effects to the same extent as has been reported for amantadine and rimantadine (Jefferson, Demicheli et al. 2006).

1.6.3 Resistance

There is no resistance to vaccines that the virus can develop except the escape strategy from the natural response to secondary infections. Antivirals however, are unchanging substances to which resistance can be developed. Most health organizations today recommend the use of oseltamivir and zanamivir. Although M2 blockers are medically approved for use, the majority of human circulating strains of influenza have grown resistant to these drugs (CDC 2008). A long period of generous use of influenza antiviral drugs and the adaptation of the viruses to cope has made the medical world more restrictive in the prescription of the more novel oseltamivir and zanamivir. Even so, resistance spreads rapidly in the new millennium (Sy, Lee et al. 2010). Media coverage of the threat of new pandemics has used the potential threat to create sensational and frightening news stories. This has dramatically increased the demand for antiviral drugs, and prophylactic use has become common. With resistance to oseltamivir on the rise, the interest for new targets for antiviral drugs has increased (Balannik, Wang et al. 2009).

1.7 Ecology

All viruses have (at least) one species with which it is constantly co-evolving. The ideal situation for a virus is when the immune response of the host is subverted, but the host does not contract acute disease from infection. Viruses that are highly adapted to humans and do not affect our fitness, gives the virus a long lived host with the opportunity to spread the virus to many new carriers. Failure to maintain such a live-and-let-live relationship would result in adverse effects for one of the parts, and regardless of which, the event will lessen the fitness of the virus. Thus, in a stable system, the virus is always near-optimal in its interaction with the host, and few mutations are truly beneficial for virus fitness, and thus the antigenic drift over time is small. New subspecies variants have no survival advantage and are thus not successfully sustained. Upon a selection changing event such as a transfer between species, where the virus is in a less than optimal state in relation to the host, more mutations allow for more rapid growth, and the genetic drift is increased.

Although recent studies show that the antigenic drift of influenza A viruses occur at a similar rate in the natural host environment as it does in temporal hosts, it is only when the virus has crossed the species barrier a highly pathogenic form have evolved (Chen and Holmes 2006). What factors in certain hosts are selective for such a trait is still unknown, but there is likely a specific pressure for this, as the same virulence have spawned many times from the same event of species transfer from dabbling ducks to chickens (Wood, McCauley et al. 1993). Influenza A virus has been found infectious to a wide range of species, but it is only among aquatic birds all 16 HA subtypes have been found (Webster, Bean et al. 1992; Fouchier, Munster et al. 2005). Infection of these animals result in no obvious signs of disease, and the infection is believed to be asymptomatic, though there is indication of ecological costs (Latorre-Margalef, Gunnarsson et al. 2009). Benign signs of disease, or asymptomatic infection points to a stable virus-host relationship, and the greater number of subtypes found indicates a long period of co-evolution. Although other birds are not considered as natural hosts, the virus crosses the species barrier relatively easy. When this has been documented, the event has been associated with a dramatic increase in virus mutation rate (Alexander and Brown 2000; Capua and Marangon 2000). These spill-over infections cause higher morbidity in the host, ranging from mild to severe / lethal (Banks, Speidel et al. 2001).

1.7.1 Host specificity

There are many factors determining whether a species can act as a host for an influenza A infection, the most obvious being sufficient contact between the host and the pathogen for infection to occur. This behavioral barrier makes some species more likely to become infected than others, and can be illustrated by the fact that since transmission of influenza A viruses occurs primarily via water, spill-over transmission to accidental hosts is mainly to species who share foraging areas with the dabbling ducks, such as waders and shorebirds (Olsen, Munster et al. 2006). For the virus to efficiently transmit between individuals of a novel host, the virus may have to change its tropism. In the case of influenza A viruses adapted to human and birds, it has been shown that the tropism is dependent on the linkage between receptor terminal SA to galactose (Gal). Influenza A viruses from humans show a preference for α2,6-linked SA and Gal, while avian isolates have a higher affinity for receptors with a α2,3-linkage (Rogers and Paulson 1983). In a laboratory setting it has been shown that isolating human adapted influenza viruses with the traditional method, of inoculating embryonated chicken eggs with virus containing material, reverts virus to α2,3 linkage preference (Katz, Naeve et al. 1987; Azzi, Bartolomei-Corsi et al. 1993; Widjaja, Ilyushina et al. 2006). Before reaching and binding to the epithelial cells, however, there are other host barriers such as mucus and alveolar macrophages to pass (Kuiken, Holmes et al. 2006). For example, in the secretions protecting the eyes and respiratory tract, different mucins containing SA are present that specifically bind and clear virus before they reach the epithelial cells. These mucins express different SA linkages in different species and also in different organ systems of the same species. Humans are better at clearing avian influenza A virus from the respiratory tract than from the eye since the mucins of the respiratory tracts are rich in α2,3-linked SA while the secretions of the eye are rich in α2,6 SA linkages. The situation is reversed in chimpanzees since their respiratory tract secretions are rich in α2,6-linked SA that make them partly resistant to infection by human influenza A virus (Olofsson, Kumlin et al. 2005). On the cellular receptor binding level there are major differences in SA linkage content between species, as well as differences within organ systems, and even between cells of the same organ systems. Taken together these differences may determine where, if at all, infection of the host may occur. Ducks that express α2,3-linked SA in the intestinal system are primarily affected by infection of the cells lining the intestinal tract (Ito, Couceiro et al. 1998). Within the human body α2,3-linked SA have been found to be predominant in the eye. In the respiratory system, α2,6 SA linkages are predominant in the upper part whilst α2,3 linkages are present in the lower part, where influenza A virus has been shown to bind preferentially to

pneumocytes type II (Shinya, Hatta et al. 2005; van Riel, Munster et al. 2006). Although not predominant, α2,3 receptors are also found on ciliated cells of the upper respiratory tract (Matrosovich, Matrosovich et al. 2004). Human influenza virus targets non-ciliated cells that express α2,6-linked SA (Matrosovich, Matrosovich et al. 2004). This information might explain why there is only limited transmission of AIV to humans, why conjunctivitis has been a common symptom, and why the respiratory infections in humans are rare but severe when they occur (Fouchier, Schneeberger et al. 2004; Beigel, Farrar et al. 2005). It was previously thought that pigs (known to express both α2,3 and α2,6-linked SA in their respiratory epithelium) were unique in their potential to act as a mixing vessel host species, where pandemic virus strains could arise by the recombination of avian and human influenza virus strains infecting the same cell (Scholtissek, Burger et al. 1985; Ito, Couceiro et al. 1998). However, the finding that both humans and chickens harbor the different receptor types in different cells indicates that theoretically this could happen in other animal hosts as well (Matrosovich, Matrosovich et al. 2004; Kim, Ryu et al. 2005). Further research has shown that although avian influenza A virus strains preferentially bind α2,3-linked SA, a further refinement of specificity exists that differs between avian species. The refinement is based on recognition of differences in the inner part of the oligosaccharide receptor (Gambaryan, Yamnikova et al. 2005). Successful attachment to a cell does not necessarily imply that infection can occur since the virus must also be able to enter the cell and cause it to replicate its genetic material. In this process the internal genes of the virus are the determinants. It has been shown that there are host specific lineages of all the different internal genes indicating species adaptation and optimization of each gene (Baigent and McCauley 2003). Some of these differences have been analyzed in detail and found to be important. For example the PB2 gene of the virus polymerase complex plays a major role. Research has shown that in avian influenza A viruses the amino acid residue 627 of the PB2 protein differs from mammalian virus strains in that; avian virus strains have a glutamic acid at this site, whereas mammalian strains have a lysine and that this is of major importance for host range restriction (Subbarao, London et al. 1993). This difference has been associated with optimal replication at different temperatures. Human influenza strains replicate in an environment of about +33 ºC in the trachea while avian strains are adapted to replication in the intestinal tracts of birds at a temperature close to +41 ºC (Massin, van der Werf et al. 2001).

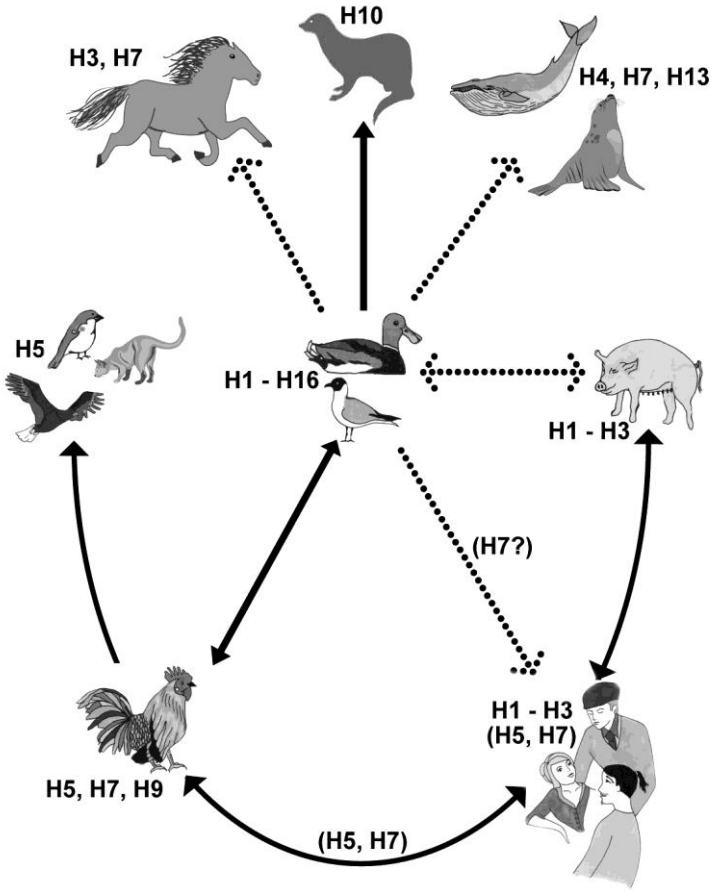

Fig 3. Illustration of the host range of influenza A virus, with the natural reservoir of influenza A virus, accidental hosts , and the subtypes that have been identified in the different groups.
Kindly provided by Rebecca Rönnmark and Eric Gisaeus.

It has also been shown experimentally that a change from glutamic acid to lysine at this site results in increased virulence for mice. The same change has also been described in H5N1 and H7N7 virus strains that have caused severe disease in humans (Hatta, Gao et al. 2001; Fouchier, Schneeberger et al. 2004). Once successful replication has taken place, the newly constructed virus must be released from the surface of the infected cell to invade new cells, and, like the HA, the NA of avian virus strains preferentially operates by cleaving the SA that are α2,3- linked, while human NAs prefer α2,6-linked SA (Baigent and McCauley 2003). Even if replication and release of new virions has been successful, there are still factors that determine whether or not the infection will remain localized in the organ of entry and if it will

prevail. First of all, the immunity of the host must be dealt with. As previously discussed; to hinder the host's innate immune response to produce interferons, that put infected cells into an antiviral state, the influenza A virus' NS1 polypeptide sequesters double stranded RNA from PKR so that the infected cell remains undetected. It has been shown that there are host specific differences in the NS1 gene of different strains, and the human NS1 gene is not optimal when introduced into mice strains (Palese 2004).
The factors determining the ability of influenza A virus to produce systemic rather than localized infection in mammals are not fully understood (Kuiken, Holmes et al. 2006). In poultry, however, the ability of the virus to invade other organs depends on whether or not the HA of the virus can be cleaved by ubiquitous extra-cellular proteases or only by specific proteases that are only present in the respiratory and gastrointestinal tract.

1.7.2 The human host

Only subtypes H1N1 and H3N2 of influenza A virus follow an epidemiological pattern in humans and are considered endemic, though a H2N2 persisted for a long time. Influenza A viruses can be isolated somewhere in the world every month and the infection is sustained and perpetuated in the human population (Cox and Subbarao 2000). The virus strains that circulate in humans mainly cause respiratory disease and preferably infect the epithelium lining the airways. Progeny virus is shed in respiratory secretions and spread effectively by airborne droplets through coughing and sneezing, and person-to-person contact. Influenza epidemics occur mainly in the winter season, from October to April in the northern hemisphere, and from May to September in the southern hemisphere. In tropical regions influenza may occur throughout the year. The seasonal fluctuations (outside of the tropics) are probably a result of factors promoting virus survival and spread, like the fact that people spend more time indoors and that the humidity is low (Nicholson 1998).
Considering the high infection rate of influenza during a seasonal outbreak, it may have a huge impact on national economies worldwide, depending on the severity of the epidemic. In temperate climates, 2-15 percent of the population becomes infected. Even during years with mild influenza epidemics, a large number of people die. In Sweden it is estimated that the number of casualties as a consequence of infection is between 1000 and 4500 depending on the strain. Many more are sick, and the cost for health care and sick leave is also high (Läkemedelsverket 2007). A spread of a subtype of influenza A virus, which the human population has not experienced before, and thus has no immunity against, may be rapid and cause concurrent outbreaks around the globe resulting in a pandemic. The severity of a

pandemic may vary with depending on the strain, and pandemic strains may behave differently to the seasonal epidemic strains. Treatment of influenza is mainly based on alleviating symptoms, although the development of NA inhibitors has created a way to shorten and perhaps limit disease if given early in the infection. Vaccination against influenza has been used for many years. In Sweden, elderly and groups at risk are encouraged to be vaccinated. Vaccination of school children, that are the principal spreaders of infection, have been tested in some countries. The main problem with vaccination is that the fast antigenic drift of influenza A virus renders the antibodies produced in response to earlier vaccinations obsolete. Therefore, in order to provide protection from disease, the vaccine has to be modified every year to adjust to the changes in the antigenic sites. Since the development and production of a vaccine takes months, qualified guesswork is used to decide which strains to include in order to match the strains of the coming season. Experimentally, AIV strains from wild birds do not replicate well in humans, and human strains do not replicate well in waterfowl (Hinshaw, Webster et al. 1983; Beare and Webster 1991). Until the outbreak in Hong Kong in 1997, the occurrence of transmission of avian strains was believed to be a rare event only causing conjunctivitis in the few affected cases (Katz 2003). However, a serological survey in rural China suggests that infection with avian subtypes has not been uncommon in people who have had close contact with domestic ducks and poultry (Shortridge 1992). In recent years, outbreaks of HPAI strains that have evolved in poultry have occurred rather frequently. The symptoms of disease and the disease pattern have been variable depending of strain. In some cases, the disease has only caused conjunctivitis and mild influenza-like illness, with no evidence of human to human spread, such as in the Canadian H7N3 poultry outbreak of 2003 where two people were affected (Tweed, Skowronski et al. 2004). In the Dutch H7N7 outbreak of 2003 conjunctivitis and influenza-like illness were also the most common symptoms, but there was also one case of fatal pneumonia. The Dutch outbreak affected at least 84 people although serological evidence suggests that as many as 1000 people were infected (Enserink 2004). During the outbreak there was also evidence of human to human transmission in some cases (Fouchier, Schneeberger et al. 2004; Koopmans, Wilbrink et al. 2004). The H5N1 outbreaks in Hong Kong in 1997 and in Eurasia and Africa 2006-2007 have caused disease in very few confirmed cases in comparison to the number of persons that have been exposed to sick birds. However, an epidemiological investigation suggests that there may be more undiagnosed cases (Thorson, Petzold et al. 2006). Low pathogenic H9N2 virus has been isolated in two children (Lin, Shaw et al. 2000) with mild influenza symptoms. H9N2 virus strains are suggested to be even more likely than H5N1 to

become the cause of a pandemic since the strains that circulate in domestic chicken and ducks worldwide have already acquired receptor specificity to prefer α2,6-linked SA (Li, Xu et al. 2003; Choi, Ozaki et al. 2004).

1.7.3 Other mammalian hosts

Influenza A virus is able to infect several mammalian species and in some cases create endemic propagation. This has been shown both experimentally and in nature as described for the species below (Hinshaw, Webster et al. 1981). Highly pathogenic virus strains, such as the currently circulating H5N1 virus that originates from South East Asia, have shown an increased host range, and are able to infect many species that had previously not been considered vulnerable. Thus, the range of species-infectivity is heavily dependent on strain type. Below, mammals which can be infected with, and transmit influenza to other individuals, will be discussed.

Suidae

Pigs are frequently infected by influenza A viruses, and there are specific swine-adapted strains. However, pigs are also susceptible both to human and avian adapted virus strains, which can be explained by the fact that the respiratory epithelium of pigs express both α2,3 and α2,6-linked SA (Ito, Couceiro et al. 1998). Avian virus strains of different subtypes have been found in pigs on a number of occasions, such as H4N6, H3N3 and a H1H1 strain in Canada (Karasin, Brown et al. 2000; Karasin, West et al. 2004). Avian H1N1 has also been isolated in China where it caused a severe outbreak in pigs 1979-1980 and has remained in the pig population since that time (Schultz, Fitch et al. 1991; Guan, Shortridge et al. 1996). Several studies have also reported human H3N2 strains in pigs after the antigenic shift in the human population in 1968 (Ito and Kawaoka 2000). Further evidence show that both avian-like and swine-like H1N1 strains circulated at the same time in pigs as well as human-like H3N2 and avian-like H9N2 (Scholtissek, Burger et al. 1983; Peiris, Guan et al. 2001). Taken together, the risk of a reassortant virus in such an environment is obvious, and several different variants have been found including reassortants between human-like H3N2 and avian-like HIN1 (Castrucci, Donatelli et al. 1993; Brown, Alexander et al. 1994; Brown, Harris et al. 1998). Humans may be infected by strains transmitted by pigs, as has been yet again obvious during the outbreak of a novel H1N1 in 2009, though direct transmission of swine-like H1N1 to humans has occurred previously, and has in some cases been fatal (Rota, Rocha et al. 1989; Claas, Kawaoka et al. 1994; WHO 2010).

Equidae

Influenza A virus strains in horses are thought to be of avian origin. Different subtypes have been found to infect horses and antigenic drift creates distinct lineages within the subtypes. At least two subtypes have created stable lineages; H7N7 and H3N8 (Berg, Desselberger et al. 1990; Guo, Wang et al. 1995; Oxburgh and Klingeborn 1999; Ozaki, Shimizu-Nei et al. 2001). Some strains have been suggested to be recent introductions from wild birds (Guo, Wang et al. 1992).

Canidae

Historically, canine species have not been a significant carrier of influenza virus. However, in 2004, an outbreak in racing greyhounds was caused by a H3N8 influenza A virus, was found to be an equine influenza A virus variant that had adapted to spread in canines (Yoon, Cooper et al. 2005). This triggered further investigation among dog breeders in the U.S and found serologic evidence of common influenza infection, and also virus isolates (Payungporn, Crawford et al. 2008). During the recent outbreak in South East Asia a surveillance investigation has isolated H5N1 influenza virus from dogs and has also found that antibodies to H5N1 are common in Thai dogs suggesting that they have previously been infected (Butler 2006).

Felidae

Feline species were not considered particularly susceptible to influenza virus prior to the recent outbreak of avian influenza H5N1 that started in South East Asia in 2003. However, after 50 captive tigers and leopards became ill and died after having been fed infected chicken carcasses several investigations were performed (Keawcharoen, Oraveerakul et al. 2004; Amonsin, Payungporn et al. 2006). It was shown that there was not only direct transmission from the contaminated food but also probable transmission between tigers (Thanawongnuwech, Amonsin et al. 2005). Experimental infection of domestic cats has shown that cats infected with the H5N1 highly pathogenic strain develop lethal systemic infection and excrete virus in both the respiratory and digestive tract secretions. The cats in the experiment could also infect each other (Kuiken, Rimmelzwaan et al. 2004; Rimmelzwaan, van Riel et al. 2006). In Europe, cats have also been found to be infected by the H5N1 virus in areas where there have been outbreaks in wild birds (ECDC 2006).

Mustelidae

Mink and ferrets have been found to be susceptible to influenza A virus and have been used in experiments since 1933 (Smith 1933; Okazaki, Yanagawa et al. 1983). When infected with human-type influenza virus, the symptoms displayed are very similar to those of humans: respiratory symptoms like sneezing and coughing, decreased apetite with following weight loss, lethargy and fever (Bodewes, Rimmelzwaan et al. 2010). As a disease model, ferrets can be argued to be the best to mimic human disease, though mice tend to be more commonly used simply because ferrets are much more difficult to handle (Maher and DeStefano 2004). Intranasal inoculation of the new A(H1N1)2009 from different strains induced very different clinical signs depending on the strain used, varying from subclinical infection to disinterest in food, fever, lethargy and severe weight loss. Mink have also been found to be naturally infected by avian influenza A virus of the subtype H10N4 during an outbreak in farmed mink in Blekinge, Sweden (Klingeborn, Englund et al. 1985). Further investigation revealed that the virus strain causing the outbreak in the Swedish mink was most likely of wild bird origin. Although the virus was very similar to avian virus strains, it was adapted to spread in-between mink (Berg, Englund et al. 1990; Englund and Hard af Segerstad 1998).

Pinnipedia and Cetacea

Infection of seals with influenza A virus has been reported on several occasions, and there is good reason to believe this is not an uncommon event. In 1979-80, seals off Cape Cod in eastern United States died of hemorrhagic pneumonia (Geraci, St Aubin et al. 1982). The causative agent of disease was found to be influenza A virus of the subtype H7N7. The virus contained avian-like genes, but behaved as a mammalian strain (Webster, Hinshaw et al. 1981). During the autopsies and handling of experimentally infected seals, people handling the animals developed conjunctivitis from influenza infection (Webster, Geraci et al. 1981). In a subsequent outbreak among seals during the season 1982-83, another even more avian-like virus was recovered from seals suffering from pneumonia. This virus belonged to the H4N5 subtype (Hinshaw, Bean et al. 1984). Further surveys of seals in the area have also found H3N3 virus strains to be present in seals (Callan, Early et al. 1995; Ohishi, Kishida et al. 2004). Seals have also been shown to be infected by influenza B virus of human origin (Osterhaus, Rimmelzwaan et al. 2000).

Whales have been found infected on several occasions, but difficulties in surveillance of these animals and high costs has made it difficult to determine the frequency of occurrence of these

events, though it does not seem common (Ridgway 1979; Hinshaw, Bean et al. 1986; Nielsen, Clavijo et al. 2001). Analysis show the most probable route of introduction has been directly from birds to the aquatic mammals (Hinshaw, Bean et al. 1986; Mandler, Gorman et al. 1990). Investigations of the receptor specificity of the SA in whale and seal lungs showed the presence of α2,3-linked SA and only a weak association with α2,6- linked SA (Lvov, Zdanov et al. 1978; Ito, Kawaoka et al. 1999; Matrosovich, Tuzikov et al. 2000). This might explain why these seals and whales are susceptible to infection by avian virus strains.

1.7.4 Influenza A virus in domestic fowl

As previously discussed, influenza A virus may enter into domestic bird populations as low pathogenic strains that only cause mild disease. Subtypes H5 and H7, however, may evolve into highly pathogenic strains. The fast mutation rate that is displayed in domestic fowl is probably due to the extremely high propagation rates in dense flocks.

Influenza A virus causes a wide spectrum of symptoms in reared birds, from mild illness to a highly contagious and fatal disease resulting in severe epidemics. Highly pathogenic avian influenza is characterized by severe illness, rapid death and a mortality in the affected populations that approaches 100 percent within 72 hours. Many different species of domestic birds including chickens, turkeys, quail and ostriches are susceptible to epidemics of rapidly fatal influenza (Capua and Mutinelli 2001; Perez, Webby et al. 2003). The main difference between infection with highly pathogenic virus strains and low pathogenic virus strains is systemic contra localized infection, the cleavage of the HA by ubiquitous proteases that expunges the restriction to cells in the respiratory tract (Suarez and Schultz-Cherry 2000). Several mutations may add to the pathogenicity of strains causing HPAI but the accumulation of basic amino acids at the cleavage site is diagnostic for disease outbreaks.

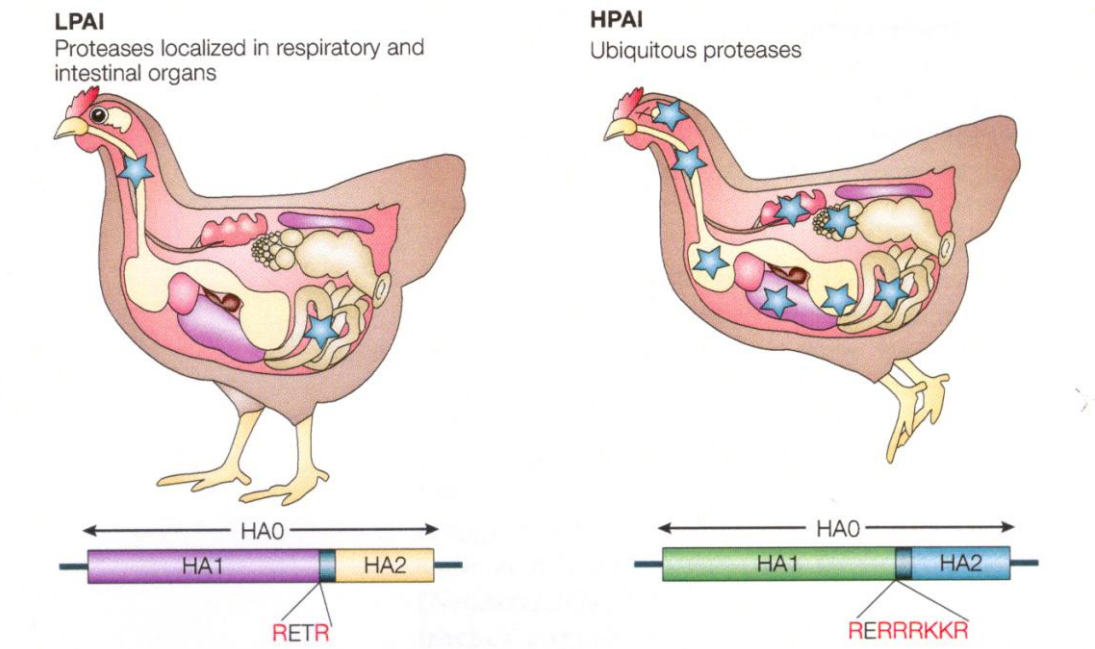

Fig 4. Illustration of localized LPAI infection vs systemic HPAI infection.
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Direct or indirect contact of domestic flocks with wild migratory waterfowl has been implicated as a cause of epizootics, but spread between farms during an outbreak is most likely caused by the movement of people and the transport of goods (Webster, Bean et al. 1992; Gilbert, Chaitaweesub et al. 2006). Outbreaks of HPAI are often difficult to control since the virus can persist and remain active for some time in the environment, and because it is highly transmissible. In areas with dense poultry populations and or limited resources for surveillance and control, such as Mexico, South East Asia and Africa, outbreaks are even harder to contain.

1.7.5 Influenza A virus in wild birds

It is widely accepted that all influenza virus strains infecting mammalian species originate from wild birds (Webster, Bean et al. 1992). All 16 HA and 9 NA subtypes been detected in isolates from avian species, and the evidence for the existence of a wild bird reservoir is strong throughout the world (Alexander 2000; Fouchier, Munster et al. 2005). It is only on Antarctica researchers have failed to isolate virus, even if serological evidence was found (Wallensten, Munster et al. 2006). On all other continents, birds have been shown to carry infectious AIV (Morgan and Westbury 1981; Austin and Webster 1993). However, most studies have taken place in developed countries and the situation in Africa, South America and parts of Asia is much less explored.

Low pathogenic influenza A virus strains have been isolated from more than 105 species from 26 different families of birds and almost all isolates come from the families *Anseriformes* and to a lesser extent *Charadriformes* and *Larida*e (Stallknecht and Shane 1988). These families include birds such as ducks, geese, swans, waders and gulls; although different species evolutionarily speaking, they share the trait of being adapted to life in an aquatic environment. Isolations of low pathogenic virus strains from pure land-dwelling birds are on the contrary rare.

Propagation

Dabbling ducks are very susceptible to, and easily become infected with, avian strains of influenza A virus through the intake of contaminated food and water. The virus remains active after passing through the low pH of the duck gizzard and infects in the cells lining the intestinal tract as well as the cells of the pulmonary epithelium (Shortridge 1992).

During the period of infection, large amounts of virus of up to 10^8 EID_{50} is shed in the duck feces, usually for about seven days, but shedding has been recorded for as long as 21 days (Webster, Yakhno et al. 1978; Kida, Yanagawa et al. 1980). Tracheal shedding also occur, though this route is probably more relevant where the social behavior of certain species makes fecal-oral transmission difficult (Ellstrom, Latorre-Margalef et al. 2008). The fact that infected birds shed high amounts of influenza A virus via fecal excretions implies that birds living in aquatic environments will contaminate the water where they live. Influenza A virus of different subtypes has been isolated in concentrations of up to $10^{2,8}$ EID_{50} /ml of water from un-concentrated lake water in lakes where wild ducks congregate (Hinshaw, Webster et al. 1979; Ito, Okazaki et al. 1995). Since the virus remains viable for some time in water, it permits transmission to other birds in the area through ingestion of contaminated water.

Influenza A virus strains were even recovered from lake water for about a month after the birds in the lake had migrated south for the winter, indicating that lakes may be a source of infection for other birds for a long time.

The species preference of influenza A virus is likely to be determined by the mode of transmission, i.e. by the fecal-oral route via water. Since virus is shed by infected birds in high quantities into an environment where it can survive for an extended time, the feeding and social behavior of the reservoir species makes it likely that susceptible individuals are exposed to the pathogen. Species that feed in shallow, calm waters, where influenza A virus is found in the highest concentrations, run the highest risk of becoming infected. Other species belonging to the *Anseriformes*, like swans and geese, graze to a larger extent on land on pastures and agricultural fields, which may lead to less efficient transmission though they are equally susceptible to the virus as their dabbling cousins. Scavenger species, such as raptors that may feed on diseased birds, are also likely to become infected, but not to take part in efficient transmission as they do not dwell in water.

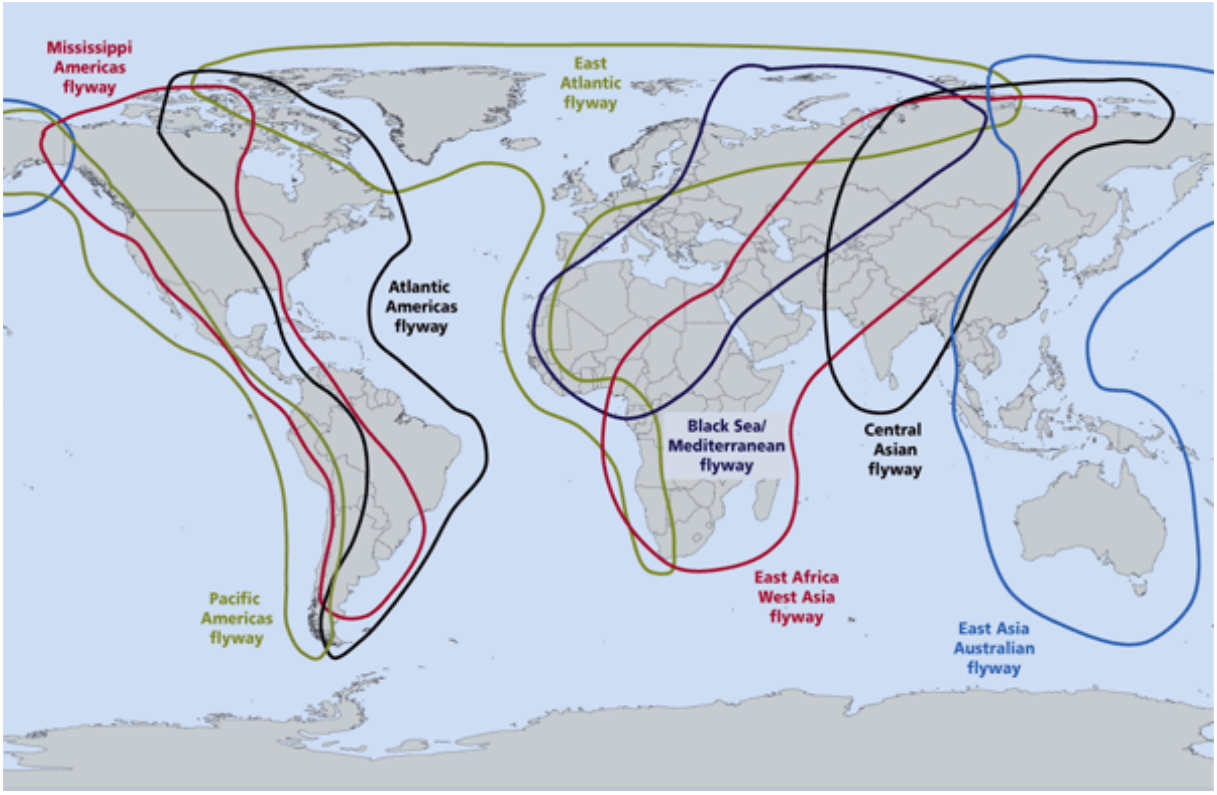

Fig 5. Overview of the major migratory flyways. Many birds who do not normally share biotope encounter one another during migration. Courtesy of Wetlands International, www.wetlands.org

The populations of *Anatidae, Charadriformes* and *Larida*e species in the world are large, the Mallard (*Anas platyrhynchos*) population by itself is estimated to 27 million birds (Kaleta, Hergarten et al. 2005). Many populations present in different areas are also connected, as these birds travel over vast distances and congregate in large numbers at staging sites during migrations (Arzel, Elmberg et al. 2006). Wild duck populations could therefore be hypothesized to support the perpetuation of even short-lived infections such as influenza, as there are enough susceptible individuals at any given time. The minimum population to support the perpetuation of the measles virus has been estimated to be 500,000 humans, and although the attack rate of influenza A virus in ducks might be lower, the population size of ducks is probably still large enough to compensate for this (Nathanson 2005). Also, certain aspects of duck demography and ecology might make the minimum population needed even smaller. First of all, the populations of ducks have high turnover rates. In Mallards about one third of the population is replaced each year, implying that this proportion is immunologically naïve (Bentz 1985; Gunnarsson, Elmberg et al. 2008). As prevalence of influenza A virus has been shown to be higher in juvenile than in adult birds, the input of juvenile, and thus immunologically naïve, birds is most likely of key importance for upholding the number of susceptible birds in the population.

One of the enigmas of influenza A virus ecology is how so many subtypes can circulate in the wild bird populations and persist from year to year, when some of these subtypes are isolated rarely, and since the prevalence in the studied bird populations differ greatly between studied species, place, and time of year. While some subtypes are frequently isolated, others have been isolated only rarely in specific places or in specific species, such as the H13 and H16 subtypes that have almost exclusively been isolated from gulls.

A clear picture of the enzootic cycle of influenza A virus in wild birds does not exist. More research is needed to elucidate the different stages involved in the interaction between the virus, the host and the environment. Hopefully, many of these variations and peculiarities will be explained in the future, when more data on the prevalence of different subtypes in different species and at different locations is available. At the present time, however, the explanations remain elusive.

Pathogenisis and immunity

It is commonly believed that all birds are susceptible to influenza A virus infection, although some species are more resistant than others. A number of variables can affect the clinical outcome of an infection; subtype, strain, host species and individual all play a role deciding the severity of the disease, which may range from non-pathogenic to lethal (Laudert, Sivanandan et al. 1993). Within the duck family there are many different species, and they may show different responses to the same infection (Suarez and Schultz-Cherry 2000). Infections by low pathogenic strains in ducks have traditionally been considered benign, as there are no evident clinical signs of disease. Though there is indication of coupling infection to a decreased body mass, aquatic birds do not appear to be severely affected by the disease and the infection does not seem to limit an infected bird's interaction with other birds or the environment (Latorre-Margalef, Gunnarsson et al. 2009). Nor does infection with low pathogenic strains seem to limit mallard capability for migration flights that could transport the virus long distances to new susceptible flocks, though the contrary has been shown for swans (van Gils, Munster et al. 2007). However, symptoms may be hard to detect. It is difficult to evaluate if the birds are completely unaffected or actually become sick in a subtle way. Few studies have been conducted in this area, but histopathological signs of mild pneumonia have been shown in ducks, even though no other signs of disease were evident (Cooley, Van Campen et al. 1989). Highly pathogenic viruses behave differently, however, and strains that are highly pathogenic for chickens may cause milder disease and different signs of disease in other species (Perkins and Swayne 2003). They may even show no signs of disease in some more resistant species of ducks and gulls (Alexander, Allan et al. 1978; Cooley, Van Campen et al. 1989; Perkins and Swayne 2002; Kishida, Sakoda et al. 2005). Due to the lack of evident effects on the birds' health status, ducks and gulls may act as carriers of some highly pathogenic strains. It is not known whether highly pathogenic influenza will revert to low pathogenicity in ducks, or whether HPAI can be perpetuated in nature indefinitely.

Studies using experimental infection have also shown that ducks may be re-infected with the same strain after two months indicating that the protection of acquired immunity is poor (Kida, Yanagawa et al. 1980). Using a recapture scheme for wild ducks and sampling the same duck at regular intervals has shown re-infection at even shorter intervals (Latorre-Margalef, Gunnarsson et al. 2009). Other bird species such as chicken, pheasant, turkey and quail mount a humoral response with high levels of IgM and IgY production (Suarez and Schultz-Cherry 2000). However, in large-scale studies, juvenile ducks are found to be infected

with influenza A virus more frequently than adult birds, indicating some sort of acquired immunity or improved immune response (Webster, Bean et al. 1992). Also, recent studies on poultry have shown a strong cross-immunity to highly pathogenic variants after vaccination with heterologous strains and that previous infection with a low pathogenic strain provides protection from disease also if the low pathogenic strain subsequently evolves into a highly pathogenic strain (van der Goot, de Jong et al. 2003; Fereidouni, Starick et al. 2009).

Evolution

Further evidence that wild birds constitute a reservoir for influenza A virus comes from studies on viral evolution, which have shown limited evolution in wild ducks over time. It has therefore been suggested that influenza A virus exists in an evolutionary stasis in the reservoir species (Bean, Schell et al. 1992; Webster, Bean et al. 1992). This suggestion is supported by analysis of strains recovered from wild ducks that have been preserved in museums since the early 20th century, which show almost no antigenic drift when compared to modern avian strains (Reid, Fanning et al. 2003). However, genetic studies on sequences from the contemporary gene pool show a mutation rate that is in contrast to the historical findings (Chen and Holmes 2006). It has also been found that co-infections of different influenza virus strains are detected less frequently in ducks than in other species, suggesting that host adapted strains prevent co-infection by other strains (Sharp, Kawaoka et al. 1997). The *Anseriformes* species is also very old, and have existed for millions of years, during which time influenza virus has obviously been able to adapt to the host in ways yet to be described (Shortridge 1992). This situation is very different from the situation when influenza A virus is introduced into mammals, an evolutionarily much younger and ill-adapted host, and avian strains that are introduced into new host species are evolving at high rates (Zhou, Shortridge et al. 1999). However, even though influenza A virus in the wild bird reservoir has been said to be in "evolutionary stasis", there have been slight changes over time. For one, different genetic lineages of influenza A virus have evolved in bird populations are distinctly geographically separated. Avian influenza A virus strains of the Americas can thus be separated from those in the rest of the world (von Hoyningen-Huene and Scholtissek 1983; Donis, Bean et al. 1989; Schafer, Kawaoka et al. 1993). A clear ecological answer to why it is so is not easy to provide, considering that summer breeding birds of Alaska spend their winters on six different continents and North American pintails birds along the West American flyway have been found to cross the strait to the Eurasian side (Miller, Takekawa et al. 2005; Winker, McCracken et al. 2007). Genetic exchange between the continents has also been shown to

occur, if at low rate (Schafer, Kawaoka et al. 1993; Makarova, Kaverin et al. 1999; Liu, Okazaki et al. 2004; Wallensten, Munster et al. 2005). Thus, the reason for such a limited exchange of AIV between the continents remains unclear. Knowledge on the interaction and spread of pathogens between different flyways and continents may be crucial in estimating the spread of AIV between different areas of the world and pre-pandemic planning.

1.8 Implications for future introductions of AIV

Low pathogenic influenza A virus strains do not seem to hinder dabbling ducks from migrating. Thus, these virus strains may be carried over large distances by the birds in a relay pattern where one bird carries the virus a short distance and another carries it further. Until the present outbreak of HPAI H5N1 that started in Asia it was not believed that wild birds could be infected with highly pathogenic virus strains and still perform long distance migrations. As some species of ducks have shown a high resistance to these strains this belief has had to be reviewed. After the HPAI H5N1 had somehow been exported to Russia, Europe and Africa in 2006, sudden satellite outbreaks in wild birds showed that transport of highly pathogenic strains by wild birds is a reality. Knowledge of influenza ecology and epidemiology thus becomes a key factor in the pre-pandemic work.

Elementary strategy planning in any battle has one basic goal: Do not to get caught off guard. The ability to foresee an outbreak allows pandemic control systems to focus containment on relevant areas in advance. Farm animals, for example, are the common interface between zoonotic diseases in the wild and humans. Primary introduction of influenza A virus into poultry and domestic animal holdings are likely due to fecal contamination by wild birds either directly by contamination of the holdings or indirectly through contaminated water supplies or feed. Holdings where wild birds and domestic birds share the same habitat due to agricultural practices are at the highest risk for outbreaks, suggesting that wild bird transmission is the most common route (Gilbert, Chaitaweesub et al. 2006). When a subtype which can be deemed a risk for humans is detected, such as the highly pathogenic H5N1, knowledge of how it may travel with migratory birds provides an opportunity for quick outbreak control. If a problematic subtype is suspected to arrive to a specific area during a specific period, regulatory government authorities may take preventive action, for example by issuing grazing restrictions for farm animals to limit their exposure to the expected pathogen.

2 Aims of this thesis

In this thesis, I place emphasis on areas which may be of importance to influenza research and in the anticipation of a novel introduction to humans.

- To investigate AIV ecology and epidemiology to increase our knowledge of how low pathogenic virus behaves in its natural setting, to facilitate the anticipation of new outbreaks.

- To describe how the natural carriers are affected by infection and to investigate whether they can be expected to behave like healthy individuals.

- To investigate alternative methods of virus isolation

- To investigate novel factors that may affect the AIV reservoir in nature, and to provide information about how this may influence future human pandemics.

3 Methodological considerations

Unfortunately, science is not perfect. Many techniques used in the laboratory are highly specific, where biology in general is not. It is constantly changing, and it is only after a change has been identified that we can adjust our methods accordingly. In this sense we will always be one step behind, unknowingly, until the novelty is brought to light. Some tests give a qualitative result which must be subjected to interpretation. Also statistical analysis of results can differ in quality, and the model algorithm may not be perfect even when it is the best available.

3.1 Screening for influenza A virus: RNA-isolation and virus detection

The screening of patient samples for the presence of influenza A virus using reverse transcription-polymerase chain reaction (RT-PCR) has been evaluated in many studies and been found to have a high specificity and sensitivity (Smith, Mock et al. 2003; Stone, Burrows et al. 2004; Hindiyeh, Levy et al. 2005). Some studies have also evaluated methods more adapted to suit the detection of AIV strains specifically (Fouchier, Bestebroer et al. 2000; Spackman, Senne et al. 2003; Spackman, Senne et al. 2003; Cattoli, Drago et al. 2004; Lee and Suarez 2004; Schlingemann, Leijon et al. 2010). The fecal samples collected for these studies were screened using RT-PCR. In brief, RNA was isolated from the samples using commercially available RNA-isolation kits according to the manufacturers' instructions, both manually and by the use of automated procedures. Subsequently, a reverse transcriptase (RT)-step creating a cDNA amplicon was carried out. Influenza A virus RNA was detected using primers directed at conserved regions of the M-gene of the influenza virus, and amplified using real time PCR technology allowing for quantification analyses and minimizing the risks of cross-contamination of samples caused by post amplification sample handling (Spackman, Senne et al. 2003). The TaqMan method uses a probe that is designed to bind in between the nucleotide sequence determined by two primers. When the polymerase replication takes place, the probe is cleaved and fluorescent light is emitted. We chose this method of real-time PCR before the alternative, SYBR-green, which uses a dye that binds unspecifically to double stranded DNA. When the PCR product determined by the primers is amplified and hybridized the dye binds and emits light. Both these methods can be used to measure the amount of the desired PCR products as the amount of light emitted is proportional to the PCR product. The amount of nucleotide template in the original sample

can also be determined, since the more template molecules present at the beginning of the reaction, the fewer cycles it takes to reach the point at which the fluorescent signal is first recorded to reach an exponential amplification phase. Using the TaqMan approach with the combination of specific primers and a specific probe is more specific than using SYBR-green technology. The SYBR-green method might have an advantage when it comes to detecting different variants of influenza A virus as it may allow for the amplification of strains with more variations in the nucleotide sequence. It is, however, less specific due to the fact that unspecific binding may occur to non-specific reaction products, including primer-dimers, and we have chosen the TaqMan technology to minimize the risk of false-positive signals.

3.2 Isolation of influenza A virus

Virus isolation was performed on all samples that were positive by RT-PCR. Egg culturing was used since it works better than culturing on existing cell lines (Nicholson 1998). Isolation of influenza viruses is traditionally performed by inoculation of ECE's with material containing viral particles. This method has been widely established as the gold standard since it was first introduced in the 1930's (Bull 1943). The WHO has issued guidelines for national surveillance programs in which embryonated chicken eggs are suggested as the preferred method of isolating influenza virus from animal samples (Stöhr 2002). Influenza viruses grow well in these vessels, but it is suspected that, like most virus isolation techniques, it introduces mutations and that there may be some difference between the original sample and the isolate on which the investigation is performed. In these studies, 200µl of the original samples was inoculated into the allantoic cavities of 11 day old ECE's. The allantoic fluid was harvested after 2 days and influenza A virus was detected by using hemagglutination assays with chicken erythrocytes. In 1951, Hirst discovered that red blood cells in suspension fail to sediment and instead agglutinate forming a lattice. Since then the hemagglutination phenomenon has been used for the detection and characterization of influenza virus (Nicholson 1998). If no influenza A virus was detected, the allantoic fluid was passaged once again in embryonated hens' eggs for one more isolation attempt. Not all of the PCR- positive samples could be isolated, indicating that there are limitations of this method. These limitations could be due to different reasons. Virus replication might require a certain amount of virus in the original sample, or the virus might not be able grow in the egg, or perhaps the RNA fragments amplified were not part of functional viruses. Another limitation when using egg culturing is that it may not always propagate all strains present in a sample. Studies have shown that more than one influenza virus strain might be present in fecal samples (Hinshaw,

Bean et al. 1980). Multiple strains cannot be told apart in the initial PCR step, and when grown on eggs, one strain might be better suited for replication and dominate the culture, in which case the other strain may not be detected.

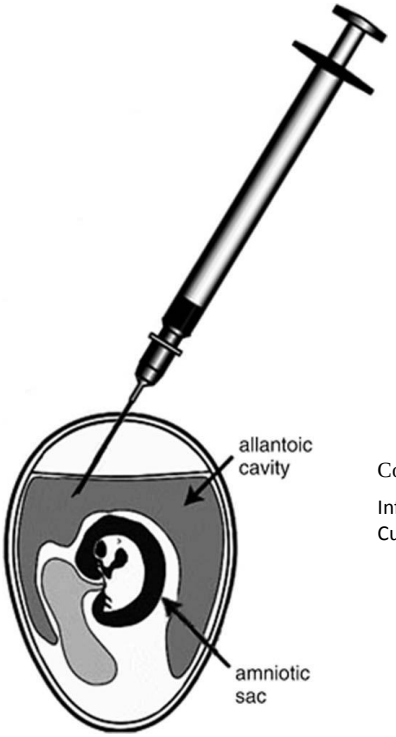

Fig 6. Virus isolation: Inoculation of sample material into the allantoic cavity of an embryonating chicken egg.

Courtesy of Dr J.Katz

Influenza: propagation, quantification, and storage.
Curr Protoc Microbiol. 2006 Dec;Chapter 15:Unit 15G.1.

3.3 Characterization of influenza A virus

Two main branches of techniques have been used to characterize influenza A viruses: serological testing using specific antibodies that can distinguish between subtypes of influenza surface proteins, and molecular analysis of the genes, which is more cumbersome but also more specific.

3.3.1 Hemagglutination inhibition and sequencing

The virus isolates found by egg culture were characterized to HA-subtype with a hemagglutination inhibition assay, using chicken erythrocytes and subtype-specific hyperimmune rabbit antisera raised against all known HA subtypes and by RT-PCR and sequencing. Virus isolates were characterized to NA-subtype by using RT-PCR and sequencing. RT-PCR was performed using primers for conserved non-coding regions for all genes (Hoffmann, Stech et al. 2001). PCR products were purified from agarose gels and sequenced using a sequencing robot.

3.3.2 Genomic analysis and phylogenetic trees

Phylogenetic trees were constructed in paper I to show the relationship between different subtypes and different strains of influenza A virus.

Alignment was performed with the Clustal W method in the Bioedit 7.0.0 software. From these alignments, neighbour joining trees for each gene and for each subtype of the surface proteins were constructed (H3, H6, H8 and N1, N3, N8, respectively) using the Mega3 software with Kimura-2 parameter (Kumar, Tamura et al. 2004).

When the trees were too large to overview with major clusters of sequences from one sampling site, representative sequences were selected from each major subclade of each tree, to represent the phylogenetic span. These representative American and Eurasian sequences were then merged and aligned with the full-length sequences from our isolates, creating uniform alignments containing 21 sequences each, and these alignments were then used to build neighbour joining trees, each tree with 500 bootstrap replicates, to illustrate the phylogenetic and geographic relationships.

4 Results and discussion

Below follows, in a brief manner, the results from the original papers included in this thesis. They will be discussed in the context of the current knowledge of avian- pandemic- and human influenza. For further information regarding details and methodology, the reader is advised to consult the original papers.

I. Reassortment between American and Eurasian Influenza Virus from waterfowl in Beringia.

Eurasian and North American AIV strains are phylogenetically distinct from each other, indicating a geographic constraint for the virus to move freely between the continents. However, it is not obvious why it should be so, considering the proximity of the continents at Bering strait. This area is a major breeding area for dabbling ducks (Northern Pintails) and that birds from the Australasian flyway may breed on the Alaskan side, sharing foraging grounds with their north American counterparts and vice versa (Winker, McCracken et al. 2007). In paper I, it is refuted that AIV prevalence is lower in the Beringia region than other breeding areas for dabbling ducks, though also in this study, as in several previous ones in the same area, there is no evidence of any virus spreading from one continent to the other (Liu, Okazaki et al. 2004; Glaser, Zamarin et al. 2006). The only genes clustering with sequences from the other side of the strait were Eurasian derived genes on the Alaskan side, but which have been recorded previously several times along the North American flyway. Though this may be due to a genetic spill-over over the strait which has established itself along the west coast of North America or an introduction from another source cannot be concluded from this study alone. Prevalence, however, is similar to what has been found in previous studies in other parts of the world, provided the focus is being put on the relevant species. Other studies have shown a much lower prevalence in this area, which may be due to several factors. In some cases, different species of birds were pooled in the calculations, allowing non-carrier species to dilute the actual prevalence in the relevant species, and many studies were conducted with crude field technology without the possibility to store samples frozen or treat them with RNA-stabilizing agents (Winker, McCracken et al. 2007). During the collection of the samples for this study, travelling with a large ship off-coast allowed the privilege of a lab and an ultrafreeze holding -80ºC where all samples were treated and stored within 2 hours of being collected.

In conclusion, the reason for the genetic separation of AIV in North America and Eurasia remains unclear. How stable this separation is also unknown, as is any potential factor that could potentially disrupt this balance. It would be prudent to remain active with the AIV surveillance of the Beringia region and investigate the movement of AIV along the migratory flyways also in the future. Knowledge of AIV ecology and its epidemiological patterns in the natural host may be key factors in the anticipation of future pandemics and pre-pandemic planning.

II. Mallard or chicken? A comparative study of influenza isolation on embryonated eggs from different species.

This article addresses the issue of mutations being introduced during virus isolation. The fact that human influenza needs to adapt to grow well in chicken eggs is widely known. Using chicken eggs for isolation of influenza has become commonplace since its first introduction in the 1930's, and the selection process for human influenza has been well described. Foremost, the virus needs to switch from its original preference for α2,6-linked SA to α2,3-linkage specificity. However, though this is the most well described difference between two species, there are others that have yet to be described. There is little known about the difference in selection pressure between different bird species, though its existence is undeniable and the effects are obvious. On numerous occasions have LPAI infected poultry farms in which the virus has undergone a dramatic phenotypic change to HPAI. As this is no single-event phenomenon, but rather quite common, at least when it comes to subtypes H5 and H7, there is obviously a specific pressure for the virus to change also after spread within the avian kingdom.

In this project, it was hypothesized that if this pressure on the virus is not of immunological origin it may still exist in the most common isolation vessel for AIV; the embryonated chicken egg. Though it was found that mallard eggs consistently yielded higher titres of virus than the chicken eggs, the mutation rate was similar, if not higher, than that found when passaging the same virus in chicken eggs. In the mallard eggs, the two viruses changed one and two aa in the primary isolation respectively, and no further mutations could be detected in the subsequent 7 passages. The same viruses behaved slightly different in chicken eggs. Both viruses changed one aa each, but one of the viruses maintained wild-type configuration for two passages before switching one aa in passage 3. Despite the fact that the material in this project is too small to conclude differences in the evolutionary rate depending on which

species of egg is used for propagation, there is no indication that mallard eggs would produce progeny virus that is more similar to the wild-type than chicken eggs.

III. Influenza Virus in a Natural Host, the Mallard: Experimental Infection Data

Discerning the virus-host interaction in the reservoir species is key information in the understanding of AIV ecology. The distinction "low pathogenic" or "highly pathogenic" are terms derived from the response to infection in chickens, where low pathogenic virus induce easilt detectable symptoms like depression, ruffled feathers, conjunctivitis, swelling and clotting of infraorbital sinuses, and subcutaneous emphysema. An autopsy of LPAI infected chickens will find pathologic signs in almost every organ. Highly pathogenic is commonly fatal with a mortality rate in chickens close to 100%. Survivability is very low. In contrast, mallards infected with LPAI show no obvious physical sign of disease and have been considered subclinical carriers of AIV. Whether they feel ill or not is hard to discern however, though feeling "under the weather" may well affect behavior and migration pattern. In this article, we address two questions highly important for the field of influenza virus ecology, i.e. (1) "Are the natural hosts of low-pathogenic avian influenza (LPAI) viruses affected by infection?" and (2) "Can the natural hosts of LPAI viruses be immune to re-infections?". To this purpose, we experimentally inoculated "wild-type" mallards (*Anas platyrhynchos*) with LPAI isolates from wild congeners. Inoculation was performed in the esophagus to mimic the natural oro-fecal cycle known to occur in this species. The novelty of our approach was to use telemetry to evaluate the effects of infection. Six juvenile mallards kept in individual cages and equipped with subcutaneous transponders and temperature loggers were monitored for body temperature, heart rate and activity before and after challenge with a LPAI H7N7 virus. Although the ducks remained alert with no modification of heart rate and activity, we recorded a moderate temperature increase in four ducks on the day they started shedding virus. This result suggests that LPAI strains may have a sub-clinical impact on their natural waterbird hosts, which may be of particular importance in the wild when these costs have to be balanced against other expenses such as growth, molt, or migration. The second originality of our study was to re-inoculate the ducks, first with a homologous subtype (the same H7N7 isolate) and then with a heterologous subtype (H5N2 isolate), to compare the successive viral shedding patterns and detect any immunity to re-infection. We found that mallards re-inoculated with a homologous LPAI subtype three weeks after primo-infection were immune to re-infection. Interestingly, immunity to re-infection by a heterologous subtype was also observed in five of the six studied birds, indicating that, in the wild, the transmission

dynamics of the different virus subtypes are not independent. Inter-individual variability was illustrated by the fact that the re-infection with H5 generated virus replicated in one duck. Our findings that LPAI viruses may have ecological costs for their natural hosts and that immunity to heterologous re-infection exists in wild birds are of significant importance. Indeed, LPAI transmission dynamics in water birds may be modified if infected birds change their behavior and if herd immunity exists in the population. Transmission models should therefore include these factors, and more studies in a natural environment conducted to discern whether this variable is associated with an ecological cost. On a broader scale, our study demonstrates the utility of using telemetry to study diseases in animals.

IV. Environmental levels of the antiviral drug oseltamivir induce development of resistance mutation H274Y in influenza A(H1N1) virus.

Antiviral drugs and passive immunization are the only options available to reduce an influenza infection once it has taken hold. Where logistical problems hinder an effective use of passive immunization, only antiviral drugs, which are not subtype specific, can be realistically used. Oseltamivir is currently the most effective agent against influenza A, and as it sterically blocks the active site of the NA protein, it is insensitive to the HA subtype of the virus. It is a cornerstone in the defense against new pandemics, to which the human population has poor resistance. However, as the active metabolite oseltamivir carboxylate (OC) is highly stable and is not degraded in purification plants for sewage water, low levels of OC can be detected in surface water where dabbling ducks forage. In this paper, we show how an OC-sensitive virus mutates under the pressure of a low level OC environment and becomes resistant. OC-sensitive virus was inoculated in mallards negative in AIV screening both in PCR and ELISA tests. The only water source was spiked with OC, and the trial was run in three sets, with different OC levels. Virus propagation to downstream generations was allowed through contact after introducing naïve mallard to the infected ones, to mimic natural transmission. Virus samples were collected on a daily basis, and known resistance mutations were detected through sequencing of the NA-gene. Resistance was verified by phenotypical analysis of NA activity over an OC gradient parallel with wild-type virus.

It could be concluded that levels of OC that can be detected in surface water today give rise to sporadic resistant strains, and at increased OC levels the resistant sub-species quickly becomes dominant. When exposing the mallards to dabbling water containing 100µg/L OC, only resistant virus could be detected after two passages.

Spread of OC resistant strains in nature increases the threat to human health from influenza A viruses. If the next pandemic is derived from a virus already resistant to OC, a significant part of the pandemic countermeasures of many countries worldwide will be rendered useless. In the past decade, the use of OC has increased steadily, and the effects on the environment and the influenza reservoir are still unknown. Continued surveillance in wild birds as a measure to understand the resistance situation in nature, and its development over time is thus of great importance. It would also be prudent to, in the light of these results, implement strategies to lower environmental levels of OC.

Concluding remarks

Future introductions of influenza viruses to humans may present us with other types of problems than what we have been forced to deal with previously. All the answers to how to act in the future may not lie in the past. Nature changes constantly, with or without our help. No matter the cause, they are all variables needed to be taken into account when planning for future outbreak control measures. In preparation for the next pandemic, it is of vital importance to understand the ecology and epidemiology of avian influenza. As previously described in the introduction, it is in the avian kingdom the pool of influenza viruses is perpetuated. The discovery of a novel subtype that may have an impact on human health or our economic system will need to be tracked and new outbreaks anticipated. Further knowledge about the epidemiology of AIV in the natural carriers can facilitate this work, knowing from the geographic location of the outbreak, and the temporal movements of carriers through this area, the direction and mannerism the infection will take, provided a spill-over back to the natural carriers occur. Keeping in mind that this mode of transport is not the only mechanism by which a pandemic may incite, it is a factor that is of vital importance to take into account for outbreak management. It is also important to increase our understanding of how influenza A virus interacts with its many different hosts, and what mechanisms exert pressure on the virus to change its characteristics.

The stability of OC is something that may become a rising problem with the increased use of oseltamivir worldwide. As the possible severity of influenza pandemics become more publicly known, the demand for prophylactic use of oseltamivir increases. If there is not a clearly stated guideline to be restrictive with the prescription of oseltamivir-containing drugs, and the threshold for the use of such drugs in lowered, increased environmental level of OC can be excepted. As shown in paper IV, low levels of OC may induce resistance in avian viruses. Even levels found today can sporadically induce resistance, and if OC levels increases further, there is indication of resistant strains becoming dominant. Considering that most plans constructed to minimize the impact of future pandemics of highly pathogenic influenza rely heavily on Tamiflu, the introduction of a highly pathogenic virus which is already resistant to OC effectively circumvents the in many cases primary defense to acutely infected patients.

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